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RAPD ANALYSIS OF GENETIC DIVERSITY AMONG AND WITHIN POPULATIONS OF *BALDUINA ATROPURPUREA* AT FORT STEWART, GEORGIA

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ABSTRACT

Compared with other federal land management agencies, the Department of Defense (DoD) has a disproportionately large number of threatened, endangered, and sensitive (TES) plant species known to occur on its lands (Flather *et al.* 1994). In some instances, this has resulted in a conflict between measures necessary to meet conservation requirements for TES species and the ability of the installation to train troops and test weapons and equipment to assure military readiness. To support the mission of the U.S. Army and Fort Stewart, researchers at the U.S. Army Construction Engineering Research Laboratories (USACERL) undertook a multi-scoped project to investigate various aspects of *Balduina atropurpurea*, a federal 'species of concern' (formerly, category 3C under the Endangered Species Act) that is state listed as 'rare' in Georgia (Smith 1994). This particular portion of the project was undertaken to determine relative levels of genetic diversity among and within on-post populations of *B. atropurpurea*. Seedlings from five representative on-post populations were evaluated using Random Amplified Polymorphic DNA (RAPD) marker analysis. Very little genetic variation was detected among or within the on-post populations evaluated. The variation observed was randomly and approximately equally distributed among populations and among individuals within populations.

KEY WORDS: *Balduina atropurpurea*, RAPD analysis, genetic diversity

INTRODUCTION

Compared with other federal land management agencies, the Department of Defense (DoD) has a disproportionately large number of threatened, endangered, and sensitive (TES) plant species known to occur on its lands (Flather *et al.* 1994). In some instances, this has resulted in conflict between measures necessary to meet conservation requirements for TES species and the ability of the installation to train troops and test weapons and equipment to assure military readiness. There is, therefore, great interest in pursuing innovative ways to manage and monitor TES species on DoD lands. To support the mission of the U.S. Army and Fort Stewart, researchers at the U.S. Army Construction Engineering Research Laboratories (USACERL) undertook a multi-scoped project to investigate various aspects of *Balduina atropurpurea* Harper, a federal 'species of concern' (formerly, category 3C under the Endangered Species Act) that is state listed as 'rare' in Georgia (Smith 1994) and known to occur on Fort Stewart.

Balduina atropurpurea (Asteraceae) is a perennial herb that occurs in wet areas of peaty pitcher plant bogs, pine flatwoods, and pine savannas with seasonal standing water (Patrick 1994). The species is endemic to the southeastern Coastal Plain area of the United States (Lutz 1995). Extant populations are known to occur in scattered locations in south to south-central Georgia, northeastern Florida, southeastern Alabama, and southern Mississippi (Helton 1995; Mississippi Natural Heritage Program 1991).

The largest, healthiest known populations of *Balduina atropurpurea* are thought to occur on the U.S. Army's Fort Stewart, Georgia, where 21 populations, distributed across five training areas, have been identified. These populations range in size from < 10 to > 2,000 individuals and cover areas of approximately 1 m^2 to 19,500 m^2 (Helton 1995; Fort Stewart Natural Resources Office, FSNRO 1996, pers. comm). Prior to 1995, only six populations of *B. atropurpurea* were known to occur at Fort Stewart. These populations were identified by The Nature Conservancy (TNC) during a survey of the installation conducted between March 1992 and October 1994 (U.S. Department of Defense 1994). An additional fourteen populations were discovered in 1995 during a survey of the installation by C. Helton (1995) and one population was identified in 1996 by Fort Stewart Personnel (FSNRO 1996, pers. comm.). These populations potentially double the number of known individuals of *B. atropurpurea* in the state of Georgia. The Fort Stewart populations are, therefore, of particular significance to the recovery and future listing status of the species.

Throughout its range, potential threats to the survival of *Balduina atropurpurea* include: alterations to the hydrological regime; loss of habitat to agricultural, commercial, and residential development; and inappropriate site management, particularly fire suppression, resulting in increased shading by shrubs and trees (Smith 1994). Military training exercises that alter the hydrological regime, cause excessive soil disturbance, or suppress the occurrence of fire could negatively impact the Fort Stewart populations. At least 43% of the Fort Stewart populations show significant impacts from tank maneuvers and/or off-road vehicle traffic. Most (>71%) of the populations are in need of prescribed burning to reduce the encroachment of shrubs and woody vegetation and to encourage the establishment of a healthy herbaceous layer. Currently, no U.S. Fish and Wildlife Service recovery plan has been prepared

for *B. atropurpurea*, and there are no existing management plans in place at Fort Stewart specifically designed for this species.

Development of a recovery plan for *Balduina atropurpurea* will be especially challenging as very little is known about the reproductive biology of this species. In the field, individuals typically produce a rosette the first year, with inflorescences produced in the second and subsequent years. Under greenhouse conditions, we observed individuals flowering during their first year of growth. Parker & Jones (1975) reported that *B. atropurpurea* is self-incompatible, and that interspecific hybridization does not occur among species of *Balduina*. They also reported the occurrence of vegetative reproduction from root stocks. R. Determann, Atlanta Botanical Garden, successfully propagated seeds of *B. atropurpurea* following four weeks of cold stratification; the majority of the seeds germinated and produced robust rosettes (R. Determann 1996, pers. comm.). Investigations into the phenology, reproduction, seed dispersal, and seedling establishment of *B. atropurpurea* are needed. Studies evaluating the effects of disturbance, as well as fire frequency and intensity, on the reproduction and health of this species are also necessary.

Knowledge regarding relative levels of genetic diversity among and within populations of *Balduina atropurpurea* at Fort Stewart would aid in determining whether any on-post populations contain unique genetic characteristics. Such populations should be given priority for conservation as their destruction would lead to the potential loss of genetic diversity necessary for adaptation to environmental changes or habitat disturbances. In addition to genetic diversity, other factors such as population health and community structure should be considered when determining the overall biological value of each on-post population.

The objectives of this study were (1) to evaluate the relative levels of genetic diversity among and within a representative sample of on-post populations of *Balduina atropurpurea*; and (2) to examine the relationship among genetic diversity, morphological diversity, and habitat diversity for this species. The information obtained from this study will aid in the development of a management plan for *B. atropurpurea* at Fort Stewart.

METHODOLOGY

Seedlings from five Fort Stewart populations of *Balduina atropurpurea* were obtained from R. Determann, Atlanta Botanical Garden. Seeds collected from the remaining populations were either immature or non-viable and failed to germinate. The five populations evaluated represent a diversity of habitats among the Fort Stewart sites in which the species is found. The seedlings were transported to Colorado State University, transplanted into pots containing a commercial, soilless potting medium (Metro Mix) and placed in a greenhouse. Six individuals each from populations '1', '2', '3', and '4', and four individuals from population '5' were included in the genetic analysis.

DNA was extracted from fresh leaf tissues according to procedures adapted from

Stewart & Via (1993) (Appendix 1). Random Amplified Polymorphic DNA (RAPD) analysis was conducted on the DNA extracts according to procedures adapted from T. Lowrey (unpubl.) (Appendix 2). When the amplification process was complete, 10 µl of electrophoresis tracking dye was added to each reaction tube and the reactions loaded into individual wells on 2.0% agarose gels. The first well on each half of the gel (upper and lower) contained a molecular weight marker of known band sizes. A negative control, without DNA, was included with each set of reactions. Gels were electrophoresed at 80 - 120 mA for approximately 16 - 18 hrs, stained with ethidium bromide for 1 - 2 hr, destained with ddH_2O for 2 - 3 hr, and photographed over UV light using Polaroid Type 665 positive/negative film. Variations in banding pattern among amplification products were analyzed from the resulting photographs.

DNA samples from four randomly chosen individuals were used for initial primer screening. Among 100 primers screened, 59 successfully amplified the DNA samples and were subsequently used in RAPD analysis on the entire set of individuals described above. Individual bands produced with each primer were numbered and scored as present or absent in each amplification product. Comparisons among and within populations were based on the presence or absence, in a given individual, of specific bands produced during amplification. To minimize potential scoring errors resulting from any uncontrollable variation in the amplification environment, only bands of moderate to high intensity (major bands) were scored for evaluation. Faint (minor) bands were excluded from the analysis. Due to the low level of variation in RAPD banding patterns detected among amplification products, quantitative analysis was not deemed appropriate, and the results were evaluated qualitatively.

RESULTS

Fifty-nine of the 100 primers initially screened produced strong amplification products and were evaluated across all samples. Of these, only seven (11.9%) revealed genetic differences among the individuals evaluated, producing a total of 25 scorable bands. Only ten of the 25 bands showed variability among samples (Figure 1). The slight differences in banding patterns produced by these primers were randomly, and approximately equally, distributed among populations and among individuals within a population. No particular individual(s) showed an especially high level of variation for RAPD banding patterns and no particular population(s) contained an especially high proportion of variable individuals (Table 1).

Thirty-one (52.5%) of the primers evaluated revealed no variation for RAPD banding patterns among samples, producing a total of 78 scorable bands (Figure 2).

Despite producing strong amplification products with the four samples evaluated during primer screening, 21 (35.6%) of the primers tested repeatedly produced a large number of non-scorable amplification products when evaluated over all samples. Fifteen (71.4%) of the non-scorable primers failed to amplify a large number of the samples and produced very faint bands among many of the samples that did amplify. High levels of non-specific amplification occurred among a majority of the samples

with two (9.5%) of the non-scorable primers. This resulted in a considerable amount of background smearing, thus preventing definitive scoring of bands. It could not be determined whether either of these two classes of non-scorable primers would have revealed variability among individuals if band scoring had been possible across all samples. No variation was observed, however, among those samples that were successfully amplified with any of these primers.

Four primers (19.0%) produced unique, repeatable banding patterns for each of the samples evaluated. These were also considered non-scorable, as they could not be used to identify relative levels of genetic variation among individuals or populations.

DISCUSSION

Despite showing strong amplification during primer screening, several primers produced non-scorable banding patterns when evaluated across populations. This was most likely due to high levels of contaminants, particularly polysaccharides, complexed with many of the extracted DNA samples (Demeke & Adams 1992; Fang *et al.* 1992). Several methods for removing the contaminants were attempted (Demeke & Adams 1992; Fang *et al.* 1992; Maniatis *et al.* 1982; Murray & Thompson 1980; Ranu 1996, pers. comm.), with varying degrees of success. To compensate for the high levels of complexed contaminants, the concentration of template DNA in each reaction mixture was reduced to 0.5 - 1.0 ng. This allowed for adequate amplification of template DNA with most of the primers evaluated, while reducing interference from the complexed contaminants. Several primers still failed to yield consistently clean amplification products across samples, resulting in either a large number of non-amplified extracts or a high degree of background smearing.

The results of the RAPD analysis indicate the Fort Stewart *Balduina atropurpurea* populations evaluated are quite similar in genetic composition. While only 25% of the Fort Stewart populations were evaluated in this study, these populations represent a diversity of the habitats found among on-post populations. The five populations chosen for genetic diversity analysis also had previously been sampled for morphological variation (D. Lincicome, unpubl. data). Once morphological analysis is complete, these two measures of diversity will be compared. An in-depth study of habitat characteristics also should be conducted for these populations and the results evaluated against morphological and genetic diversity. Site and population data obtained thus far include population size; approximate number of individuals per population; associated herbaceous, shrub, and tree species; soil type and nutrient content; evidence of fire or disturbance; and general site quality (Helton 1995; unpubl. data). The majority of the site and population data have not yet been analyzed. Soil sample analyses revealed similarities among the on-post sites. The Fort Stewart populations occur on slightly acidic (pH 3.9 - 5.0) soils with a sandy loam texture and a relatively low organic matter content (1.0 - 6.5%). Soil nutrient content varied considerably from site to site, particularly levels of phosphorus, potassium, and iron (unpubl. data).

Figure 1. Banding patterns from amplification of *Balduina atropurpurea* DNA using RAPD analysis. Amplification products show variation among DNA extracts. Both inter- and intra-population variation is evident. Lanes 1 and 22: molecular weight marker; Lanes 2-7: DNA extracts from population 1; Lanes 8-13: DNA extracts from population 2; Lanes 14-19: DNA extracts from population 3; Lanes 20-21 and 23-26: DNA extracts from population 4; Lanes 27-30: DNA extracts from population 5; Lane 31: negative control (no DNA).

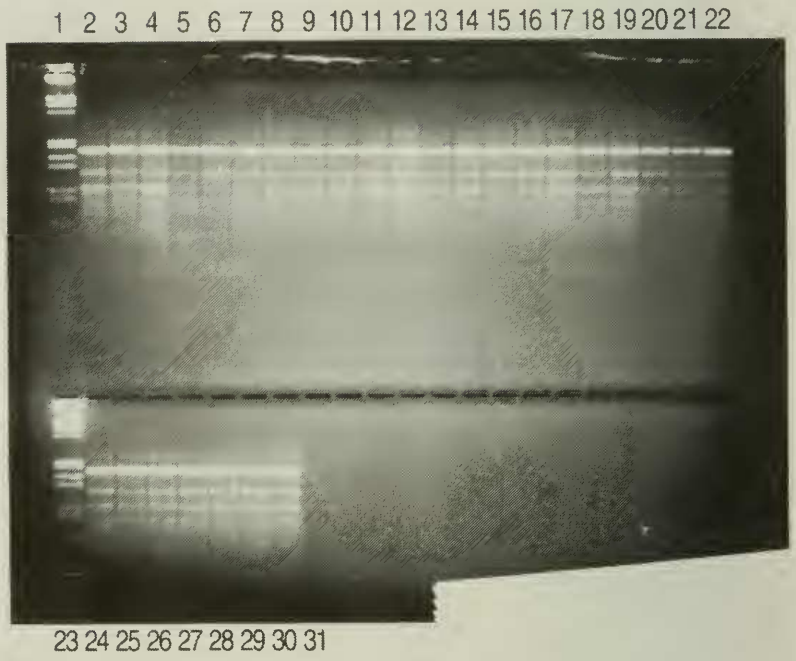

Figure 2. Banding patterns from amplification of *Balduina atropurpurea* DNA using RAPD analysis. Amplification products show no variation among DNA extracts. Lanes 1 and 23: molecular weight marker; Lanes 2-7: DNA extracts from population 1; Lanes 8-13: DNA extracts from population 2; Lanes 14-19: DNA extracts from population 3; Lanes 20-22 and 24-26: DNA extracts from population 4; Lanes 27-30: DNA extracts from population 5; Lane 31: negative control (no DNA).

Although the morphological analysis is not yet complete, initial evaluations revealed differences among the on-post populations for several vegetative and seed characters (D. Lincicome, unpubl. data). The variance observed for those characters, however, is high, thereby reducing differences among character means for the populations (D. Lincicome 1996, pers. comm.). Thus, levels of morphological variation within and among populations may be similar. This is consistent with the results from the RAPD analysis. While little genetic variation was detected overall with RAPD analysis, the variation that was observed occurred with similar frequency both among and within populations.

If considerable inter- or intra-population variability for morphological characters should ultimately be found, this would not necessarily reflect the presence of high levels of genetic variation among or within populations. Morphological variation is a product of differential gene expression and may not be correlated with underlying levels of genetic variation. Populations may be very similar in genetic composition, yet show considerable morphological variation. This can result from differences in phenotypic expression in response to environmental differences (Williams *et al.* 1995) or from interactions among a small number of genes controlling morphological characters (Kochert *et al.* 1991).

Given the out-crossing nature of *Balduina atropurpurea*, it was not unexpected to observe genetic variation within populations, as well as among populations. Many questions remain unanswered regarding the reproductive biology of this species including identification of the primary pollinator(s), the distance viable pollen can "travel" between populations, and the origin(s) of the on-post populations. Thus, the degree to which populations might be genetically differentiated from one another cannot be predicted. Among the populations evaluated, population '5' exists in a distinct location relative to the others and would have the lowest probability for gene exchange with surrounding populations. Population '5', however, did not show a high proportion of genetic variation relative to the other populations evaluated nor was it genetically distinct from the remaining populations based on RAPD banding patterns.

It would be beneficial to evaluate the remaining sixteen on-post populations using RAPD analysis to determine whether any of these populations contain unique genetic characteristics. Such analyses would be time-consuming and expensive given the low levels of genetic variation detected among the populations evaluated thus far. In the short term, conducting in-depth habitat characterizations for each population, in combination with evaluations of reproductive success and population sustainability, would aid management decisions. Ideally, genetic evaluations should occur simultaneously with habitat characterization studies since long-term survival of the species is ultimately dependent upon maintaining adequate genetic diversity among and within populations. Genetic diversity provides a species with a means for better adaptability to environmental changes and/or habitat disturbance.

To obtain a better understanding of the relative value of the Fort Stewart populations compared to surrounding populations, we recommend that habitat characteristics, population parameters, morphological variation, and genetic diversity also be evaluated for several off-post populations and compared to results obtained from the on-post populations. A diverse range of populations should be sampled, including nearby off-post populations in Tattnall and Bulloch counties, Georgia, as

well as more distant populations known to occur in Georgia, Florida, Alabama, and Mississippi. This would aid in the development of a recovery plan for *Balduina atropurpurea* within the region as a whole, in addition to a management plan for the species at Fort Stewart.

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APPENDIX 1
Methodology used for DNA extractions.
Adapted from Stewart & Via (1993).

For each sample, approximately 0.1 g of fresh leaf tissue was harvested and mechanically ground in an individual tissue grinder. Five µl β-mercapto-ethanol and 1 ml warm (60°C) CTAB extraction buffer (2% w/v CTAB, 1.42 M NaCl, 20 mM TRIS-HCl pH 8.0, 2 % w/v PVP, and 5 mM ascorbic acid) were added, and the mixture incubated in a 60°C water bath for 30 min. Each sample was transferred to a clean eppendorf tube and 500 µl chloroform:isoamyl alcohol (24:1) was added. The samples were placed on a mechanical shaker at 100 rpm for 15 min., followed by centrifugation at 10,000 rpm for 5 min. The upper phase of each sample was transferred to a fresh eppendorf tube using a pasteur pipette and re-extracted with chloroform:isoamyl alcohol. The DNA was precipitated out of each sample by adding an equal volume of ice-cold (approximately 0°C) isopropanol to the tube and gently inverting the mixture. Samples were placed in a -20°C freezer overnight to further precipitate the DNA. This was followed by centrifugation at 5,000 rpm for 5 min. The supernatant was discarded and the pellets washed with 500 µl of a 0.2 M sodium

acetate/70% ethanol solution. The sodium acetate/ethanol mixture was added to the tube, and the pellet was dislodged and allowed to soak for 10 min. The samples were briefly centrifuged and the supernatant discarded. The pellets were air-dried and resuspended in 200 µl Tris-EDTA. Extracted DNA samples were stored at -20°C.

The quantity of DNA obtained per sample was measured using a spectrophotometer, based on relative absorption of 260 and 280 nm wavelengths UV radiation passing through the sample. The quality of the DNA extracted from each sample was determined by electrophoresing a subsample through a 1.0% agarose gel, staining the gel with ethidium bromide, and exposing the gel to UV light.

APPENDIX 2
Methodology used for RAPD analysis.
Adapted from T. Lowrey, University of New Mexico (unpubl.).

Each RAPD reaction mixture was prepared by adding the following reagents to a sterile microcentrifuge tube: 17 µl sterile ddH_2O, 5 µl Master Mix [10× Electrophoresis Reaction Buffer (Boehringer Mannheim); 10 mM each dATP, dCTP, dGTP, and dTTP; ddH_2O; and 1 M $MgCl_2$ (magnesium chloride), (bringing the total $MgCl_2$ concentration to 2 mM)], 1 µl (5 picamoles) primer (Operon Technologies, Inc., Alameda, California), and 1 µl diluted DNA sample (0.5 - 1.0 ng). The reaction mixture was gently vortexed, then briefly centrifuged to collect the mixture at the bottom of the tube. Each reaction mixture was overlain with approximately 50 µl electrophoresis grade mineral oil to prevent evaporation during amplification. The reaction tubes were placed into individual wells, to which one drop of mineral oil had been added, in a DNA thermal cycler (MJ Research Inc. PTC 100 Programmable Thermal Controller). The amplification program used was as follows: (Step 1) 'Hot Start' of 2 min. @ 94°C; (Step 2) addition of 0.5 unit Taq DNA polymerase (Boehringer Mannheim) to each reaction tube, @ 80°C (held for 20 min.); (Step 3) time delay of 3 min. @ 94°C; (Step 4) 35 cycles, each consisting of 1 min. @ 94°C (denaturing), 1 min. @ 38°C (first annealing), 30 sec. @ 54°C (second annealing), 2 min. @ 72°C (elongation); (Step 5) 15 min. @ 72°C (final elongation); (Step 6) indefinite soak @ 4°C.

Table 1. Variation in RAPD Banding Patterns Among and Within Populations of *Balduina atropurpurea*.[1,2]

Primer # OPZ-7	
Pattern (+++)	I (B1, B7, B11, B13); II (B22, B35, B38); III (B41, B58, B67); IV (B70, B75, B79); V (B85, B88)
Pattern (+-+)	I (B3, B14); II (B26, B34, B39); III (B46, B49, B64); IV (B71,

B74, B76); V (B87)

Primer # OPZ-9*
Pattern (++) I (B1, B14); II (B34); III (B58, B64); IV (B76, B79); V (B82)
Pattern (+-) I (B3, B7, B11, B13); II (B22, B26, B35, B38, B39); III (B41, B46, B49, B67); IV (B70, B71, B74); V (B85, B87, B88)
* B75 missing (did not amplify with this primer)

Primer OPT-20*
Pattern (++++++) I (B1, B3, B7, B14); II (B26, B35, B38, B39); III (B58, B67); IV (B71, B75, B79); V (B85, B87, B88)
Pattern (++++-+) I (B11, B13); II (B34); III (B41, B46, B49, B64); IV (B70, B74, B76); V (B82)
* B22 missing (did not amplify with this primer)

Primer OPAL-18*
Pattern (+++) I (B7); II (B26, B34); III (B46, B64, B67); IV (B71); V (B85, B88)
Pattern (+-+) I (B3, B11, B13, B14); II (B35, B38, B39); III (B41, B49, B58); IV (B70, B74, B75, B76, B79); V (B87)
* B1, B22, and B82 missing (did not amplify with this primer)

Primer OPA-7*
Pattern (+++) I (B13); III (B46, B58); IV (B76)
Pattern (++-) I (B1, B3, B7, B14); II (B22, B26, B34, B35, B38, B39); III (B41, B49, B64, B67); IV (B70, B71, B74, B75, B79); V (B82, B85, B87, B88)
* B11 missing (did not amplify with this primer)

Primer OPJ-13
Pattern (+++) I (B1, B3, B7, B11, B13, B14); II (B22, B26, B34, B35, B38, B39); III (B41, B46, B49, B58, B64, B67); IV (B70, B71, B74, B79); V (B82, B85)
Pattern (+-+) IV (B76); V (B87)
Pattern (-++) IV (B75); V (B88)

Primer OPJ-10*
Pattern (+++++) III (B46, B58); IV (B70, B74, B76)
Pattern (++++-) I (B3, B7, B11, B13); II (B22, B26, B35, B39); III (B41, B49, B64); IV (B71, B75, B79); V (B82, B85, B87)
Pattern (+++-+) I (B1)
Pattern (++-+-) II (B38)
* B14, B34, B67, and B88 missing (did not amplify with this primer)

1 I - V refer to population numbers.
2 B1, B3, etc. refer to DNA extracts from individual plants evaluated in RAPD analysis.